
P-3639P1/BDIS-3CIP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicants | : | Maino *et al.* |
| Serial No. | : | 08/803,702 |
| Filed | : | February 21, 1997 |
| For | : | METHOD FOR DETECTING T CELL RESPONSE TO SPECIFIC ANTIGENS IN WHOLE BLOOD |
| Group Art Unit | : | 1644 |
| Examiner | : | Phillip Gambel, Ph.D. |

Bethesda, MD

Hon. Assistant Commissioner
for Patents
Washington, D.C. 20231

**DECLARATION OF CALMAN P. PRUSSIN, M.D.**
**UNDER 37 C.F.R. § 1.132**

Sir:

I, CALMAN P. PRUSSIN, M.D., declare as follows:

1. I am the Head of the Clinical Allergy Unit, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health, a post I have held since 1996. I am the recipient of the National Institutes of Health Director's Award and United States Public Health Service Commendation Medal, both for my research at NIH. My *curriculum vitae* is attached hereto as Exhibit A.

2. I have no financial interest in, and do not consult for, the assignee of the above-referenced application.

The opinions set forth in this Declaration are not made in my capacity as a government employee and are not intended to represent the views of the United States Government.

3. I have been asked to address two issues relating to the above-identified patent application: (1) whether the invention would have been obvious to one of ordinary skill in the art at the time the invention was made, and (2) whether the specification disclosure, at the time of its filing, enabled the skilled artisan to practice the claimed methods with antibodies specific for other than the explicitly named cytokines. As further described below, I was engaged in, and published on, the measurement of intracellular cytokines by flow cytometry before the December 1996 priority filing of the present application, and I today routinely use the claimed methods as the principal tool in my continuing research.

4. To prepare this declaration, I have reviewed the specification of the present application, applicants' response of March 1999 – including the presently pending claims – the outstanding office action of March 2000, all references cited by the Examiner in the official action of March 2000, and the additional references cited herein. I have also relied upon my own experience in measuring intracellular cytokine expression by flow cytometry.

The State of the Art

5. Before answering either of the questions posed to me, it is perhaps best to describe the state of the art prior to the filing of the present patent application and its parent. Six references are particularly relevant. Because I understand that all but one have been considered by the Examiner, I will discuss them only briefly.

6. In 1993, Jung and colleagues[1] pioneered the use of flow cytometry for detecting cytokine production at the single cell level in peripheral blood mononuclear cells ("PBMC"). Prior to this publication, cytokine production had typically been assessed either in bulk, *e.g.* by ELISA measurement of cytokines secreted into medium, or on the single cell level after *in vitro* culture, *e.g.* by limiting dilution or enzyme-linked immunospot assays (LDA and ELISPOT, respectively).[2] Alternative single cell methods for examining small numbers of cells included *in situ* hybridization of nucleic acid probes to cytokine mRNA within cells that had been fixed and permeabilized on a microscope slide and, analogously, binding of anti-cytokine antibodies to cytokine proteins within cells fixed and permeabilized on a microscope slide. The Jung method made detection by flow cytometer feasible, making possible multiparametric detection of cytokine production by large numbers of individual peripheral blood cells.

7. In the Jung *et al.* method, PBMC are stimulated with a polyclonal stimulus, PMA+I,[3] in the presence of monensin, an inhibitor of the Golgi-mediated secretion pathway. After stimulation, the cells are fixed and permeabilized, and the cytokine that had accumulated intracellularly then labeled with an anti-cytokine antibody. A second step, fluorophore-

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[1] Jung *et al.*, "Detection of intracellular cytokines by flow cytometry," *J. Immunol. Methods* 159:197-207 (1993).

[2] The use of ELISPOT to identify antigen-induced cytokine secretion is described further below with respect to the two Lolli *et al.* references cited by the Examiner: *FEMS Immunol. Med. Microbiol.* 7:55 - 62 (1993) and *AIDS Res. Human Retrovir.* 10(2):115 - 120 (1994).

[3] Phorbol 12-myristate 13-acetate (PMA) plus ionomycin (I) ("PMA+I").

labeled anti-Ig antibody is added, and the cells are then analyzed by flow cytometry.

8. Jung *et al.* demonstrated that polyclonal stimulation in the presence of a secretion inhibitor provided sufficient intracellular accumulation of IL-2, IL-4 and IFN-γ in a sufficient percentage of T lymphocytes to permit detection of each these cytokines by flow cytometer.

9. For reasons further discussed below, it is important to note that Jung *et al.* demonstrated flow cytometric detection of cytokines that had earlier been demonstrated to be produced by T lymphocytes, and to do so used anti-cytokine antibodies that had already been produced for other purposes.

10. In both May[4] and December[5] 1995, I published papers describing an improvement on the Jung *et al.* method. Jung *et al.* had used a two-step labeling: the primary anti-cytokine antibody had been unlabeled; a fluorophore-conjugated secondary antibody, specific for the first, had then used to render the cytokine fluorescently detectable. By using anti-cytokine antibodies directly conjugated to fluorophores, as well as by improving the fixation and permeabilization approach, we both simplified the protocol and significantly improved the signal to noise ratio.

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[4] Elson *et al.*, "Flow cytometric analysis for cytokine production identifies T helper 1, T helper 2, and T helper 0 cells within the human $CD4^{+}CD27^{-}$ lymphocyte subpopulation," *J. Immunol.* 154(9):4294-4301 (May 1, 1995) (Exhibit B).

[5] Prussin *et al.*, "Detection of intracytoplasmic cytokine using flow cytometry and directly conjugated anti-cytokine antibodies," *J. Immunol. Methods* 188:117-128 (1995).

11. I would like to make three points about our 1995 publications.

12. First, the cytokines that we detected had earlier been shown to be produced by activated T cells. Furthermore, although some of the fluorophore-antibody conjugates that we used to detect these cytokines were custom conjugates, we did not ourselves raise any of these antibodies. In fact, our earliest pilot studies were performed with antibodies that had been developed for use in detecting cytokines by ELISA, with hybridoma supernatants, and even with polyclonal antibodies.

13. Second, like Jung *et al.*, we used PMA+I as a polyclonal stimulus, because PMA and ionomycin activation are the most robust stimuli for most lymphokines, providing the best conditions for observing intracellular cytokine staining.

14. Third, we demonstrated that we could detect IL-5 in addition to the cytokines detected by Jung: IL-2, IL-4, and γ-IFN. With respect to IL-5, we particularly demonstrated that cytokine flow cytometry is useful in examining the increased IL-5 production characteristic of eosinophilic states and that IL-5 production is limited to the CD27 negative population.

15. Between the May and December publications of our work, Picker *et al.*[6] published similar work. I understand that the Picker *et al.* reference is the principal reference applied by the Examiner in his obviousness rejection.

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[6] Picker *et al.*, "Direct Demonstration of Cytokine Synthesis Heterogeneity Among Human Memory/Effector T Cells by Flow Cytometry," *Blood* 86:1408-1419 (August 15, 1995).

16. I will address Picker *et al.*'s data further below; here, I would like to comment on certain aspects of their methodology.

17. First, as did we and Jung *et al.*, Picker *et al.* detected cytokines earlier shown to be produced by T cells, using antibodies that they did not themselves raise. Like our group, Picker *et al.* used antibodies directly conjugated to fluorophores.[7]

18. Second, like Jung *et al.* and our group, Picker *et al.* used PMA+I as "a potent activating stimulus."[8] Picker *et al.* further demonstrated that another "accessory cell independent" pan-T activating stimulus, a combination of anti-CD3 and anti-CD28, could be used, as could "accessory cell dependent" stimulation by superantigen.

19. Finally, alone among the three groups, Picker *et al.* used concurrent detection of and gating on the early activation antigen CD69 to increase the apparent sensitivity of their method, and uniquely among the three groups used brefeldin A, rather than monensin, as the secretion inhibitor.

20. The four references described above – Jung *et al.*, Elson *et al.*, Prussin *et al.*, and Picker *et al.* – are all directed to the application of flow cytometry to the detection of intracellular cytokine production by activated T lymphocytes. As such, they are clearly relevant to a method that is presently claimed as "[a] method of detecting antigen-

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[7] Although Picker *et al.* note that they also used a series of fluorophore-unconjugated monoclonal antibodies to confirm the staining specificity of the direct conjugates.

[8] Picker *et al.* p. 1411, col. 1.

specific cytokine production by individual T lymphocytes," the final step of which is "flow cytometrically detecting the intracellular binding of . . . [a] cytokine-specific antibody by cells in . . . [a] defined T lymphocyte subset."

21. The next two references[9] use enzyme-linked immunospot (ELISPOT) technology to detect antigen-specific T cells. Although the ELISPOT technique does not visualize cytokines intracellularly, these references are also relevant to the methods claimed in the present application, since the ***ultimate*** goal of the claimed methods – and indeed, the reason we and other cellular immunology groups use the claimed methods – is to detect antigen-specific T cells. I understand that the claims of the present application are being amended to make the ultimate goal of detecting antigen-specific T lymphocytes more clear.

22. In the ELISPOT technique, peripheral blood mononuclear cells are cultured in the presence of antigen, and antigen-specific T cells are thereafter identified on a single cell basis by virtue of their secretion of γ-IFN. In the Lolli *et al.* CMV reference, for example, peripheral blood mononuclear cells are incubated with CMV antigen for 76 hours, then plated into wells of a microtiter plate having a nitrocellulose bottom coated with anti-human γ-IFN. After an additional 16 - 20 hours, the cells are removed and γ-IFN that captured locally on the nitrocellulose visualized using a secondary antibody. In the Lolli *et al.* AIDS reference, peripheral blood mononuclear

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[9] Lolli *et al.*, "T and B cell responses to cytomegalovirus antigens in healthy blood donors and bone marrow transplant recipients," *FEMS Immunol. Medical Microbiol.* 7:55 - 62 (1993) ("Lolli *et al.* CMV reference"); Lolli *et al.*, "HIV antigen-reactive T cells detected by antigen-induced interferon γ secretion," *AIDS Res. Human Retrovir.* 10:115 - 120 (1994) ("Lolli *et al.* AIDS reference").

cells are cultured with HIV antigen for 76 hours directly in the ELISPOT plate, after which time cells are removed and the immunospecifically captured IFN-γ visualized.

Differences between claimed invention and the prior art

23. At the conceptual level, the methods claimed in the present patent application differ from those of Jung *et al.*, Elson *et al.*, Prussin *et al.*, and Picker *et al.* in applying flow cytometric detection of intracellular cytokines to the detection of antigen-specific T cells, rather than to detection of broad T helper subsets. At the procedural level, this conceptual difference is manifested in the stimulation of peripheral blood mononuclear cells with MHC-dependent nominal antigens, rather than by polyclonal stimuli.

24. The methods claimed in the present application differ from the ELISPOT approach used by Lolli *et al.* in a variety of conceptual and procedural respects: in the detection of cytokines intracellularly, rather than after secretion; in detection of antigen-specific T cells directly *ex vivo*, rather than after significant culture and *in vitro* expansion; in the large numbers of cells that can be tested in an automated device, in contrast to the small number of spots that are detected and counted visually; in the ease with which data can be collected, versus the time-consuming and tedious ELISPOT approach; in the multiparametric nature of the flow cytometric method – that is, in the ability to measure multiple parameters, such as multiple cytokines and/or multiple surface markers in a single assay – in contrast to the dedicated single parameter determination afforded by ELISPOT. I discuss several of these differences in more detail below.

Lack of suggestion or reasonable expectation of success

25. In my opinion, the disclosure of Picker *et al.* (and, equivalently, the disclosures of Jung *et al.*, Elson *et al.*, and Prussin *et al.*) would not have suggested to one of ordinary skill in the art[10] that flow cytometric methods of detecting intracellular cytokines should be applied to the detection of antigen-specific T lymphocytes, or that if applied, such approach would have a reasonable expectation of success. Quite to the contrary, I believe that others of ordinary skill in the art would have read the Picker *et al.* reference, as I had done, to suggest quite the **opposite**, that the procedure **could not** be successfully applied the detection of antigen-specific T lymphocytes.

26. As noted above, I have reviewed applicants' response of March 1999. I agree with the argument set forth in the response that the percentage of cytokine-producing T cells observed by Picker *et al.* in the setting of a fully activating stimulus would have taught away from the methods now claimed, given the expectation at that time regarding the frequency of antigen-specific T lymphocytes expected in PBMC. I would, however, focus attention on a different set of numbers from those cited by the applicants in their response.

27. To argue, as applicants did, that the percentage T lymphocytes responsive to specific antigen would have been expected to be far smaller than the percentage responsive to polyclonal stimuli states only half the problem, for if it were simply a matter of detecting rare events, more cells could be run through the cytometer. The other half of the problem lies

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[10] By "one of ordinary skill", I refer to a cellular immunologist having at least a graduate degree (Ph.D., M.D., or equivalent) and having laboratory experience with flow cytometry of lymphocytes, with ELISPOT, or with limiting dilution assay (LDA), if not all three.

in the amount of noise in the detection system: the reason Picker *et al.* teach away from the present invention is that the frequency then expected of true antigen-specific events is not sufficiently above the level of noise reported in the system to suggest reliable detection.

28. To see this most clearly, I direct the Examiner's attention to Picker *et al.*'s Figure 1 (page 1410). Looking at the middle panel of the top row, fully 0.7% of $CD8^-$ ($CD4^+$) cells and 1.1% of $CD8^+$ cells give a positive signal for IL-2 (middle panel), **even without stimulation**. Looking at gamma interferon (right-most of the Picker *et al.* FIG. 1 panels), fully 0.8% of unstimulated $CD8^-$ ($CD4^+$) cells give a positive signal for γ-IFN, even without stimulation. This is a measure of noise in the Picker *et al.* system; in order to be detected reliably using the Picker *et al.* technique, a true event would need to be present in greater than about 1% of $CD4^+$ or $CD8^+$ T cells.

29. Turning, then, to the Lolli *et al.* references, which seem representative of ELISPOT reports in the art of the time, the median frequency of antigen-specific T cells reported is on the order of 0.01% of PBMC (about 10 IFN-γ secreting T cells per $10^5$ PBMC). Even accounting for the disparate denominators, these numbers are far below the level of noise reported by Picker *et al.*[11]; they would, therefore, be presumptively undetectable.

30. There is, furthermore, substantial *in vitro* clonal expansion in the methods described by Lolli *et al.*

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[11] And close to the level of noise observed in our own work: although not fully comparable, our own false positive rates ranged from about 0.01 - 0.1%, Prussin *et al.*, p. 122, cols. 1 - 2.

Assuming a doubling time of about 12 hours, Lolli's 72 - 96 hour incubation effects an expansion of about 50 to 150 fold in antigen-specific T cells prior to detection. The ELISPOT data of Lolli *et al.* thus argued particularly strongly against adapting the Picker *et al.* method for detection of antigen-specific T lymphocytes in samples freshly drawn from peripheral blood.

31. To reiterate, nothing in Picker *et al.* would have suggested that the percentage of antigen-specific T cells could reliably be detected by a technique, flow cytometric measurement of intracellular cytokine, that has a noise level as high as 1.1%. Indeed, Picker *et al.*'s numbers, particularly in light of numbers provided by Lolli *et al.*, would have suggested that antigen-specific T lymphocytes **could not** be detected, teaching away from the present invention.

32. I was, accordingly, quite surprised when I first heard of Louis's results; I hadn't thought it possible. And I think that this was the general reaction among those of ordinary skill in the art of cellular immunology.

Adoption by those in the art

33. Today, my lab uses the claimed methods as a major tool in all aspects our research. For example, we have used intracellular cytokine detection of antigen-specific T cells to demonstrate that vaccination with plasmid DNA encoding a specific leishmanial antigen is more effective than vaccination with leishmanial protein plus recombinant IL-12 in eliciting long-term immunity capable of controlling *L. major*

infection.[12] As another example, we recently used intracellular cytokine detection of antigen-specific T cells to demonstrate that high numbers of HIV-specific $CD8^+$ T cells exist even in patients with high-level viremia and progressive disease.[13] Applying the techniques to granulocytes as well as T cells, we recently demonstrated that early in the response to specific allergens, the IL-4 and IL-13 response by T cells is far lower than that by basophils, demonstrating that basophils are the predominant peripheral blood cells that express IL-4 and IL-13 in the first 6 hours after activation by allergen antigens.[14]

34. None of the studies mentioned in the preceding paragraph would have been possible using the methods of Jung *et al.*, Elson *et al.*, Prussin *et al.*, or Picker *et al.*, which do not identify antigen-specific T cells; the studies are made possible by the invention described and claimed in the present application.

Cytokines

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[12] Gurunathan *et al.*, "Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection," *Nature Med.* 4:1409 - 1415 (December 1998) (attached hereto as Exhibit C).

[13] Gea-Banacloche *et al.*, "Maintenance of large numbers of virus-specific $CD8^+$ T cells in HIV-infected progressors and long-term nonprogressors," *J. Immunol.* 165:1082 - 1092 (2000) (Exhibit D). I would note in passing that the our introduction to this paper states explicitly that prior art techniques, LDA in particular, substantially underestimate the frequencies of antigen-specific T cells, confirming the substance of applicants' March 1999 response.

[14] Devouassoux *et al.*, "Frequency and characterization of antigen-specific IL-4- and IL-13-producing basophils and T cells in peripheral blood of healthy and asthmatic subjects," *J. Allergy Clin. Immunol.* 104:811-819 (1999) (Exhibit E).

35. I understand that the Examiner also questions whether the claimed methods of detecting antigen-specific T lymphocytes can, without undue experimentation, be practiced by detecting other than the explicitly named cytokines. The answer is yes.

36. There are really two questions here: the first is whether the skilled artisan would readily be able to identify cytokines, other than those specifically named in the present specification, that can serve as a signal of antigen-specific T cell stimulation; the second is whether, for those cytokines that can serve as indicators of antigen-specific T cell stimulation, one skilled in the art would be able to detect them in the claimed methods.

37. With respect to the first of these questions, it is certainly true that not all cytokines are expressed by T lymphocytes. It is equally true, however, that the skilled cellular immunologist would be able easily to determine which are so expressed.

38. The simplest method, of course, would be to look in the literature to see which cytokines are known to be expressed by T lymphocytes.

39. As I mentioned above, Jung *et al.*, Elson *et al.*, Prussin *et al.*, and Picker *et al.* all chose to detect cytokines that had earlier been shown to be produced by activated T lymphocytes, collectively exemplifying measurement of IL-2, IL-4, IL-5, and IL-13. Picker *et al.*, describing earlier published work, further disclosed that TNFβ, IL-6 and IL-10 are produced by activated T lymphocytes. Still other references had reported the expression of additional cytokines by

activated lymphocytes. For example, Andersson *et al.*[15] reported that 19 different cytokines could be detected immunocytochemically, adding IL-1α, IL-3, IL-8, GM-CSF, G-CSF, TNFα, TGF-β1-3 and IL-1 receptor antagonist to the list of cytokines known at the time to be expressed by activated lymphocytes.

40. I will not here exhaustively review the cytokine literature. It suffices here to note that the skilled immunologist would readily have been able, in late 1996, early 1997, to identify cytokines not explicitly named in applicants' specification that would be expected to be expressed by activated T lymphocytes and that would, accordingly, be suitable indicators of antigen-specific T cell stimulation.

41. Turning, then, to the second of the two questions, I have already noted above that neither Jung *et al.*, Picker *et al.*, nor we had to raise our own antibodies. Nor, typically, would one attempting to use a "new" cytokine as a marker of antigen-specific stimulation in the methods of the present invention.

42. Typically, to adapt the methods of the present invention to detection of a cytokine known to be expressed by activated T cells, but not yet reported to have been detected intracellularly after antigen-specific stimulation, one would obtain three to four distinct antibodies already known to be specific for that cytokine, and test each in turn in the present method to see which gives the best results; it would be simply a matter of routine optimization, varying the antibody and antibody concentration to optimize the signal to noise

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[15] Andersson *et al.*, "Concomitant *in vivo* production of 19 different cytokines in human tonsils," *Immunology* 83:16 - 24 (1994) (Exhibit F).

ratio. Given the high level of skill in the art, I would venture that such optimization would be routine for most, if not all, cellular immunologists. If the antibody were not available directly conjugated to fluorophore, these pilot experiments could be done, as in the early days, using a secondary antibody for two step immunofluorescence detection. Again, such two stage labeling is a matter of routine in cellular immunology labs.

43. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and may jeopardize the validity of the application or any patent that issues thereon.

11/20/00
Date

Calman P. Prussin, M.D.

7 August, 2000

# CURRICULUM VITAE

**Name: Calman Philip Prussin**

**Citizenship:** United States of America

## Education and Training

1975-1980 - B.A. in Chemistry. University of California San Diego, La Jolla, California

1980-1984 - M.D. University of Southern California School of Medicine, Los Angeles

1984-1988 - Internal Medicine Residency, Los Angeles County-University of Southern California Medical Center, Los Angeles, California

1989-1991 - Medical Staff Fellowship, Laboratory of Cellular and Molecular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

1991-1995 - Allergy and Immunology Clinical Fellowship, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

## Chronology of Employment

1990-date - Commissioned Officer, U.S. Public Health Service.
Rank: Commander

1996-date - Head, Clinical Allergy Unit, Laboratory of Allergic Diseases, NIAID

## Board Certification

Diplomate, American Board of Internal Medicine, 1988
Diplomate, American Board of Allergy and Immunology, 1993

## Society Memberships

American Academy of Allergy, Asthma and Immunology
American College of Physicians
American Thoracic Society

## Honors and Awards

Provost's Honor List: 1975, 1976, 1977
President's Undergraduate Fellowship, 1978
Honors in Infectious Diseases Clerkship, 1984
National Institutes of Health Director's Award, 1998
United States Public Health Service Commendation Medal, 1999

## Grant Review Panels

Chair, Adult AIDS Clinical Trial Group Monokine Research Study Section, 1997
Member, Clinical Sciences Special Emphasis Panel, National Center for Research Resources (Flow Cytometry), 1998
National Academy of Sciences/ U.S.A.I.D Cooperative Development Research Grant Review Committee, 1999
Asthma and Allergy Foundation of America Grant Review Panel, 1999

## Editorial Boards

Clinical Immunology Newsletter, 1996-1998

## Journal Review

Clinical and Experimental Allergy
Clinical Immunology and Immunopathology
Cytometry
International Archives of Allergy and Immunology
Journal of Allergy and Clinical Immunology
Journal of Clinical Immunology
Journal of Immunological Methods
Journal of Immunology
Journal of Leukocyte Biology
Nature Medicine

## Professional Contributions

National Institutes of Health
Chair , NIAID Institutional Review Board, 1996-1997
Member, NIAID Institutional Review Board, 1995-1997
Member, NIH Human Subjects Research Advisory Committee, 1996-1997
AIDS Clinical Trial Group, Flow Cytometry Committee, 1995-1996
Member, Steering Committee, NIH Cytokine Interest Group, 1996-1998
Chair, Methods in Cytokine Biology Symposium, NIH Cytokine Interest Group, 1996, 1998, 2000
Attending, Allergy and Immunology Consult Service, 1994-date
Member, Graduate Medical Education Committee, 1999-date
Member, NIAID Clinical Research Standards Implementation Committee, 2000
Course Lectures Taught: Immunology 504, 1997-date;
Immunology 517, 1999-date

American Academy of Allergy, Asthma and Immunology
Fellows in Training Committee, 1995-97
Bronchoalveolar Lavage Committee, 1995-1998
Immunotherapy of Asthma Committee, 1996-1998
Training Program Director's Committee, 1999-date

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7. **Prussin C.** and B. Foster. 1997. TCR Vα24 and Vβ11 coexpression defines a human NK1 T cell analog containing a unique Th0 subpopulation. *J Immunol* 159:5862-5870.

8. Gurunathan S., K. Irvine, C-Y Wu, J. Cohen, E. Thomas, **C. Prussin**, N. Restifo and R.A. Seder. 1998. CD40 ligand trimer enhances both humoral and cellular immune responses and induces protective immunity to infectious and tumor challenge. *J Immunol* 161:4563-4571.

9. Gurunathan S., **C. Prussin**, D.L. Sacks and R.A. Seder. 1998. Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. *Nature Medicine* 4:1409-1415.

10. Devouassoux G., D.D. Metcalfe, **C. Prussin**. 1999. Eotaxin potentiates antigen dependent basophil IL-4 production. *J Immunol* 163:2877-2882.

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15. Stobie L, Gurunathan S, **Prussin C**, Sacks DL, Glaichenhaus N, Wu CY, Seder RA. 2000. The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. Proc Natl Acad Sci U S A. 97:8427-32.

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2. **Prussin C** and M. Kaliner. 1996. Anaphylaxis. *Allergy, Asthma and Immunol* 1: 3-6.

3. **Prussin C**. 1997. Cytokine Flow Cytometry: Understanding cytokine biology at the single-cell level. *J Clin Immunol* 17:195-204.

**Book Chapters**

1. **Prussin C**. and M.A. Kaliner. 1996. Anaphylaxis. In: Immunobiology; Principles of Medical Biology, E.E. Bittar and N. Bittar, eds. JAI press, Greenwich, CT. 231-238.

2. **Prussin C**. and P. Openshaw. 1998. Detection of intracellular cytokines by flow cytometry. In: Current Protocols in Immunology, J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober and R. Coico, eds. John Wiley & Sons, New York, NY. 6.24.1-6.24.11.

3. **Prussin C**. and D. Metcalfe. 2000. Update on the Management of Asthma. In Current Therapy in Internal Medicine, A.S. Fauci, ed. Mosby, Saint Louis , MO. In press.

**Abstracts**

1. **Prussin C**. and M.A. Kaliner. 1994. Single cell assay of interferon-□ and interleukin-4 producing CD4 lymphocytes in atopic subjects. *J Allergy Clin Immunol* 93: 233.

2. **Prussin C**., L.H. Elson, T. Nutman and D.D. Metcalfe. 1995. IFN-γ and IL-5 are produced by mutually exclusive subsets of human CD4 cells, *J Allergy Clin Immunol* 95: 222.

3. **Prussin C**., B. Foster and D.D. Metcalfe. 1996. IL-2 is Produced by Both Th1 and Th2 Cells, *J Allergy and Clin Immunol* 97: 299.

4. Rabin R.L., I. Fuss, **C. Prussin**, J. McDyer, O. Garraud, T.B. Nutman, T.A. Fleisher, R. Seder, W. Strober and S.M. Holland. 1996. Characterization of an immunodeficiency manifest by severe viral skin infections, eczema, and asthma. *FASEB Journal* 10: (6) 372-372.

5. **Prussin C**. and B. Foster. 1997. Vα24+, Vβ11+ natural T cells are the human analog of murine NK1.1 T cells and demonstrate a predominant Th1 phenotype, *J Allergy Clin Immunol* 99:S257.

6. **Prussin C.**, B. Foster and D.D. Metcalfe. 1998. Basophils are the predominant IL-4 producing peripheral blood cells both in vivo and in vitro, *J Allergy Clin Immunol* 101:S41.

7. Devouassoux G., B. Foster, L.M. Scott, D.D. Metcalfe and **C. Prussin**. 1999. Frequency and characterization of antigen specific IL-4 and IL-13 producing basophils and T cells in peripheral blood of normals and asthmatics, *J Allergy Clin Immunol* 103: S194.

8. **Prussin C**. and B. Foster. 2000. Asthmatic and healthy control subjects demonstrate differential T cell responsiveness to ubiquitous aeroallergens. *J Allergy Clin Immunol*, 105: S338.

9. Foster B., L.B. Schwartz, D.D. Metcalfe and **C. Prussin**. 2000. Tryptase is expressed by human basophils independent of disease status. *J Allergy Clin Immunol*, 105: S88.

P-3639P1/BDIS-3CIP

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | |
|---|---|---|
| Applicants | : | Maino *et al.* |
| Serial No. | : | 08/803,702 |
| Filed | : | February 21, 1997 |
| For | : | METHOD FOR DETECTING T CELL RESPONSE TO SPECIFIC ANTIGENS IN WHOLE BLOOD |
| Group Art Unit | : | 1644 |
| Examiner | : | Phillip Gambel, Ph.D. |

Atlanta, GA

Hon. Assistant Commissioner
for Patents
Washington, D.C. 20231

**DECLARATION OF JOHN D. ALTMAN, Ph.D.**
**UNDER 37 C.F.R. § 1.132**

Sir:

I, JOHN D. ALTMAN, Ph.D., declare and state as follows:

1. Having devoted considerable effort to problems that require the identification and enumeration of antigen-specific T cells, I have been asked to address the question whether the methods for detecting antigen-specific T cells described and claimed in the above-referenced patent application

would have been obvious in late 1996 and early 1997 to those of us who were skilled in cellular and molecular immunology.

2. The short answer is that the methods described and claimed in the instant application revolutionized the field of cellular immunology, solving a long-sought but unmet need. Nothing in the art of which I was personally aware at the time, and nothing certainly in the references cited by the patent Examiner, would have suggested or motivated the line of inquiry pursued by Skip Maino and Louis Picker; nothing in my own experience, and nothing certainly in the references cited by the patent Examiner, would have intimated that such an approach would be attended by a reasonable expectation of success. Indeed, much of the art taught away from the present invention, rendering the results all the more surprising.

3. I am presently an Assistant Professor of Microbiology and Immunology at Emory University School of Medicine, a Member of the Emory Vaccine Center, and a Pew Scholar in the Biomedical Sciences. I neither consult for nor have financial interest in the assignee of the present invention. My *curriculum vitae* is attached hereto as Exhibit A.

4. My lab at Emory addresses issues of T cell response to viruses and to antiviral vaccines. In collaboration with Peter Doherty's laboratory at St. Jude Children's Research Hospital, for example, we have used murine γ-herpesvirus infection (a model for human infection by Epstein-Barr virus and human herpesvirus 8) to clarify the roles of antigen-specific $CD4^+$ and $CD8^+$ T cells during the lytic, latency, and reactivation phases of infection. For example, we have shown that even though antigen-specific $CD8^+$ T cells may be present at

the time and site of virus challenge, establishing a high level of $CD8^+$ T cell memory to lytic-phase epitopes alone does not protect against the longer-term consequences of γHV infection.[1] We have also shown that virus-specific $CD8^+$ T cell numbers are maintained during γ-herpesvirus reactivation in CD4-deficient mice.[2] More recently, we demonstrated that postexposure vaccination increases the prevalence of γ-herpesvirus-specific $CD8^+$ T cells, but confers minimal survival advantage on CD4-deficient mice.[3] In other work, we have studied the virus-specific T cell responses through the development, effector, and recovery phases of primary and secondary influenza pneumonia.[4]

5. Critical to all of this work is the ability to detect and accurately to enumerate antigen-specific T cells.

6. My interest in measuring antigen-specific T cell responses dates back to my postdoctoral work in Mark Davis's lab in the Department of Microbiology and Immunology at Stanford Medical School. In the 1980s, the Davis lab cloned the genes of the TCR β, TCR α, and TCR δ subunits. By the time of my arrival in the early 90's, Mark's lab was applying this knowledge to basic questions about the antigen-specificity of T cell responses.

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[1] Stevenson *et al.*, *Proc. Natl. Acad. Sci. USA* 96:9281-9286 (August 1999) (attached hereto as Exhibit B).

[2] Stevenson *et al.*, *Proc. Natl. Acad. Sci. USA* 95:15565-15570 (December 1998) (attached hereto as Exhibit C).

[3] Belz *et al.*, *Proc. Natl. Acad. Sci. USA* 97(6):2725-2730 (March 14, 2000) (attached hereto as Exhibit D).

[4] Flynn *et al.*, *Proc. Natl. Acad. Sci. USA* 96:8597-8602 (July 1999) (attached hereto as Exhibit E).

7. These efforts are perhaps best exemplified in McHeyzer-Williams *et al.*, "Antigen-specific development of primary and memory T cells in vivo,"[5] in which the Davis lab relied upon the known uniformity in TCR αβ usage in the murine response to pigeon cytochrome C, along with homogeneity in the length of the third complementarity-determining region (CDR3) of TCR subunits as a surrogate marker for antigen-specific T cells. I think that two points can reasonably be inferred from the extreme efforts, including the use of single cell PCR, that were undertaken in this paper to detect and enumerate antigen-specific T cells: first, there was tremendous motivation in the field to develop methods that would permit the detection and accurate enumeration of antigen-specific cells at the single cell level; and second, there were no methods then readily available that met this need.

8. It was against this backdrop that I undertook with Mark and Mike McKeyzer-Williams to develop a better method. The result, described and claimed in U.S. Patent No. 5,635,363 (attached hereto as Exhibit G), uses tetrameric MHC molecules, complexed to peptide, to stain T cells that are specific for the peptide/MHC complex. Detection and enumeration is then performed by flow cytometry. Peter Doherty, 1996 Nobel Laureate in Medicine or Physiology, has been gracious enough to describe the tetramer technology as one of two technological advances that has revolutionized analysis of immunity mediated by $CD8^+$ T cells.[6]

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[5] *Science* 268:106-11 (April 1995) (attached hereto as Exhibit F).

[6] Doherty, *Curr. Opin. Microbiol.* 1:419-422 (1998) (attached hereto as Exhibit H).

9. The other "involves short-term (5-6 hours) stimulation of T cells with viral peptide in the presence of Brefeldin A, followed by fixation and staining for cytoplasmic interferon-γ (IFN-γ),"[7] which I understand to be an embodiment of the invention described and claimed in the instant patent application.

10. It should be clear that as motivated as we were to develop methodology to detect and enumerate antigen-specific T cells, we did not ourselves invent the methods pioneered by Maino and Picker. And the same can of course be said of the large number of other highly motivated groups working in this field.

11. I have read the office action mailed March 27, 2000 with respect to the instant application and I have reviewed the references cited therein by the Patent Examiner. I have also read the specification and pending claims of the instant application, and applicants' response of March 1999. In my opinion, none of the references cited by the Examiner, alone or in combination, would have motivated the person of ordinary skill in our art[8] to attempt the methods presently claimed.

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[7] Doherty, *supra*, at 420, col. 2.

[8] I have been asked to identify the credentials of this hypothetical person of "ordinary skill in the art". I would say that the person of ordinary skill has at least a Ph.D., if not further postdoctoral training, in some aspect of immunology, likely cellular immunology, and has experience in culturing and enumerating lymphocytes.

12. The Examiner cites Picker *et al.*[9] as a primary reference in his obviousness rejection. Correctly noting that "Picker et al. differs from the instant methods by using polyclonal activators or superantigen stimulation," the patent Examiner then concludes that "the ordinary artisan was [therefore] well motivated to apply various analyses to detect antigen specific activation of T cells." Office Action at 5.

13. As I have already noted in the paragraphs above, I agree that "the ordinary artisan [would have been] . . . well motivated to apply various analyses to detect antigen specific activation of T cells": we were indeed ourselves highly motivated. However, this generalized motivation to solve the problem is by no means the same as having been motivated by the Picker *et al.* disclosure to solve the problem of detecting antigen-specific activation of T cells by modifying the Picker *et al.* technology itself.

14. A major reason is that Picker *et al.*'s results were consistent with the then-prevailing wisdom that the number of antigen-specific T lymphocytes would be too low to detect by flow cytometry; consistent with the prior art understanding, nothing in the Picker *et al.* reference itself would have suggested that the assay be adapted to measure antigen-specific T cells. It would, in fact, have argued against such modification.

15. Polyclonal mitogens and superantigens stimulate a far greater percentage of T lymphocytes than do MHC-dependent

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[9] Picker *et al.*, "Direct Demonstration of Cytokine Synthesis Heterogeneity Among Human Memory/Effector T Cells by Flow Cytometry," *Blood* 86:1408-1419 (1995).

nominal antigens. Yet even after the "fully activating stimulus"[10] by polyclonal mitogen (PMA+I),[11] Picker observed that only **2.5**% of $CD8^-$ lymphocytes, and only **0.8**% of $CD8^+$ cells, could be shown to express IL-4. Analogously, after stimulation of **20.9**% of $CD4^+$ cells by superantigen, only **1.8**% of the $CD69^+CD4^+$ cells could be shown to express IL-4 (Picker Fig. 3). IL-2 and γ-IFN were produced in a higher percentage, but still only in a subset of the activated T cells.

16. Prior art assays, particularly limiting dilution assays ("LDA"), had suggested that the percentage of T cells that would respond to nominal antigen would be far lower than the percentages Picker *et al.* observed responding after a "fully activating stimulus". It was reasonable, therefore, for the person of ordinary skill in immunology, upon reading the Picker *et al.* paper, to assume that the intracellular cytokine assay would be unable to detect and reliably report the number of antigen-specific T cells.

17. The Examiner emphasizes, however, that Picker *et al.* measure intracellular cytokine synthesis on a single cell basis. Although true, that fact is beside the point: the issue is not whether one could detect an individual event, ***should it be present***, but rather whether a sufficient number of such individual events would be expected to be present upon

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[10] Picker *et al.*, page 1417, col. 1.

[11] Picker *et al.*'s figure 1 shows that, after 4 hours' incubation with PMA+I and Brefeldin A (BFA), fully **94.7**% of $CD8^-$ (that is, $CD4^+$ cells) become $CD69^+$, indicating activation; Picker *et al.*'s figure 1 further shows that **96.4**% of $CD8^+$ lymphocytes become $CD69^+$ after incubation with PMA+I and BFA.

stimulation with MHC-dependent nominal antigen as to render such events readily detectable by flow cytometry. Picker *et al.*'s results, coupled with the then-prevailing wisdom, would have predicted that they could not.

18. We now know, based in part upon use of the very assay claimed in the instant application, and based in part also upon use of our tetramer assay, that LDA substantially underestimates the number and percentage of antigen-specific T cells. Although we may have suspected that LDA provided an underestimate, we did not then suspect the magnitude of such undercounting. I think that it is quite telling that Peter Doherty entitles his 1998 *Current Opinion* review "***The New Numerology*** of Immunity Mediated by Virus-Specific $CD8^+$ T Cells".[12]

19. The Examiner's reliance upon Picker *et al.*'s use of cells from delayed-type hypersensitivity (DTH) sites (Office Action p. 6) to argue otherwise is misplaced. Rather than evincing an attempt by Picker *et al.* to detect T cells specific for the immunogen, *i.e.* mumps antigen, Picker *et al.*'s efforts are directed instead "[t]o determin[ing] whether the ability of

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[12] See Exhibit H.

That said, I must take issue with the unqualified nature of the language in applicants' March 1999 response that "the LDA data and ELISPOT data in the prior art were **wrong**." It really depends on what you expect assays such as the LDA to report: if you expect LDA to report the frequency of cells that proliferate and ultimately give a score in the LDA, clearly it's not wrong, that's what the data are; if, on the other hand, you expect LDA to report the frequency of circulating T cells that are antigen-specific before extensive *in vitro* culture, then the data indeed provide a substantial underestimate.

$CD4^+$ memory/effector T cells to heterogeneously produce the cytokines under study was a function of the nature of the activating stimulus, the presence or absence of accessory cells, or the site from which the T cells were obtained. . . . " Consonant with this goal, stimulation for purpose of assay was with superantigen, not the MHC-dependent mumps antigen itself (Picker *et al.*, pages 1413-1414).

20. In summary, despite strong motivation in the art to develop assays that would permit the detection and enumeration of antigen-specific T cells, neither we nor others in the art were motivated to adapt the Picker *et al.* intracellular cytokine technology to such detection. The principal reason was that the Picker *et al.* disclosure itself, consistent with prior art data, taught away from its successful modification, suggesting that too few antigen-specific events would be present to permit their ready and reliable detection.

21. Turning, then, to the Examiner's comment that "[b]oth Lolli et al. references teach and provide an expectation of success in detecting antigen-specific responses, including T cells respons[ive] to CMV antigen, including measuring the production of cytokines (e.g. γIFN) from single T cells," Office Action at page 5, that does not really speak to whether there would have been any expectation of success in detecting antigen-specific responses, including T cells respons[ive] to CMV antigen, including measuring the production of cytokines (e.g. γ-IFN) from single T cells" using the flow cytometric techniques of the present invention. Additionally, the Examiner has overlooked an essential methodologic aspect of Lolli *et al.*'s ELISPOT approach that would teach away from the present invention.

22. In each of the two Lolli *et al.* references, there is substantial *in vitro* culture of the lymphocytes prior to detection. In the measurement of T cell responses to CMV antigens,[13] for example, the authors culture the cells 76 hours before placing them in the ELISPOT plate and then culture in the ELISPOT plate for additional 16 - 20 hours (p. 57, col. 2). In the measurement of T cell responses to HIV antigens,[14] Lolli *et al.* culture the cells 76 hours directly in the ELISPOT plate (p. 116, col. 2). In each case, those skilled in the art would have assumed that the purpose of the culture was to expand the number of antigen-specific T cells prior to detection. Far from speaking to a reasonable expectation of successfully adapting the Picker *et al.* technology to detection of antigen-specific T cells, the two Lolli *et al.* references instead suggest, consistent with Picker *et al.* and prior art results, that antigen specific T cells cannot readily be detected without antecedent amplification of their number.[15]

23. Thus, I disagree with the Examiner that the two Lolli *et* al. references would have suggested to one of ordinary skill in the art of cellular immunology in late 1996 or early 1997 that adapting the Picker *et al.* technology to detection of

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[13] Lolli *et al.*, *FEMS Immunol. Med. Microbiol.* 7:55 - 62 (1993).

[14] Lolli *et al.*, *AIDS Res. Hum. Retrovir.* 10(2):115-120 (1994).

[15] The Lolli *et al.* references particularly teach away from the whole blood embodiments of applicants' invention. Not only do the references suggest that necessity of *in vitro* culture, but both further teach the depletion of monocytes prior to the *in vitro* culture.

antigen specific T cells would have been attended by a reasonable expectation of success.

24. In short, I disagree with the Examiner that one of ordinary skill in the art, at the time the instant application was filed, would have found the invention to have been obvious.

25. I would also argue that the rapid and widespread embrace of this assay by cellular immunologists around the world is evidence of the degree to which the invention provided an elegant solution to a long felt need in the art.

26. Flow cytometric measurement of intracellular cytokine production has rapidly become a required technique in cellular immunology. We, and I would venture to say most others in the field of cellular immunology, now routinely use flow cytometric measurement of intracellular cytokine synthesis to detect antigen-specific T cells. We use it as a complement to tetramer staining;[16] other labs use it exclusively.

27. To conclude, the invention described and claimed in the instant application revolutionized cellular immunology, providing an elegant and now widely adopted solution to a long-felt problem. Had the invention indeed have been obvious, then I expect that others of us, as highly motivated as we were to develop means to detect antigen-specific T cells, would be among the named

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[16] *See, e.g.*, Stevenson *et al.*, *Proc. Natl. Acad. Sci. USA* 96:9281-9286 (August 1999) (Exhibit B); Stevenson *et al.*, *Proc. Natl. Acad. Sci. USA* 95:15565-15570 (December 1998) (Exhibit C); Belz *et al.*, *Proc. Natl. Acad. Sci. USA* 97(6):2725-2730 (Exhibit D).

inventors; we of course are not, and I am thus pleased to offer these comments in support of the present inventors' nonobvious contribution to the art.

28. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and may jeopardize the validity of the application or any patent that issues thereon.

11/29/2000

Date

John D. Altman, Ph.D.

# CURRICULUM VITAE

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DOB: October 6, 1962 (Detroit, Michigan)
Citizenship: United States.

## PROFESSIONAL EXPERIENCE:

Assistant Professor of Microbiology and Immunology,
Emory University School of Medicine (January 1997-present)

Member, Emory Vaccine Center (January 1997-present)

## EDUCATION:

Stanford University

- Post Doctoral Fellow (September 1991-1996)
- American Cancer Society Post Doctoral Fellowship ( 1991-1994)
- Research: Detection of antigen specific T cells by flow cytometry using novel preparations of multimeric MHC/peptide complexes; direct *ex vivo* description of the phenotype of HIV-specific $CD8^+$ T cells; negative selection of peptide-antigen-specific thymocytes.
- Supervisor: Mark M. Davis

University of California, San Francisco

- Degree: PhD. in Pharmaceutical Chemistry 1991
- Thesis title: Engineering of Bovine Pancreatic Trypsin Inhibitor for NMR and Protein Folding.
- Supervisor: Dr. Irwin D. Kuntz

Massachusetts Institute of Technology

- Degree: SB. in Chemistry
- September 1980- June 1984
- Research Supervisor: Dr. Michael A. Marletta

## AWARDS AND FELLOWSHIPS:

- Pew Scholar in the Biomedical Sciences 1999-2003
- Post Doctoral Fellow of the American Cancer Society -- 1991-1994
- University of California Regents Fellow -- 1987-1988
- National Science Foundation Graduate Fellow -- 1984-1987
- Phi Beta Kappa -- MIT 1984
- American Institute of Chemists Student Award -- 1984
- University of Michigan Regents Scholarship -- 1980

## CONSULTANTSHIPS:

- Beckman/Coulter Corporation, 1997-present.
- Merck & Co., Inc. 1998-present
- St. Jude Childrens Medical Center, 1999-present

## SOCIETY MEMBERSHIPS:

- American Association of Immunologists, 1998-present

## GRANT SUPPORT:

### As Principal Investigator:

1RO1AI4373-01 12/1/97-12/1/2001 30%
NIH $615,186 (Direct costs over 4 years)
T Cell Repertoires Specific for Defined Viral Epitopes

1R01AI42518-01 1/1/98-12/31/2001 40%
NIH $613, 393 (Direct costs over 4 years)
HIV-Specific $CD8^+$ T Cells: Frequencies and Phenotypes

P01-AI43045-010003 01/01/98-12/31/02 10%
NIH $750,000 (Direct costs over 5 years)
Macaque MHC and Motif Analyses

N01-AI-85332: 85332-05 9/21/98-9/20/01 5%
McKesson BioServices $2,356,480 (Total costs over 3 years)
The MHC Tetramer Core Facility

McKesson BioServices is the Prime Contractor for the NIH-AIDS Research and Reference Reagent Program with NIAID. The MHC Tetramer Core Facility is a subcontract to that contract.

**As Coinvestigator:**

1R21A144359-01 ZAI1 9/1/98-8/31/2000 5%
NIH $300,000 (Direct costs over 2 years)
Functional Analysis of HIV-1-Specific CTL –Target Conjugates
Principle Investigator: Jeff Safrit, Yerkes Regional Primate Research Center

**Subcontracts**

1 R01 AI46719-01 3/15/1999-2/29/2004 3%
NIH $17,818
In vitro stimulation of HIV Specific $CD8^+$ T Cells

We will provide MHC tetramer reagents to Dr. Peter Katsikis at MCP/Hahnemann, the principle investigator of the grant, for use in cell free antigen-specific stimualtion experiments. We will also consult with him about the design of his experiments and the use of tetramers. There is no scientific overlap with any of our studies.

1 P01 AG11915-06 7/1/1999-6/30/2004 0%
NIH $20,000
Dietary Restriction and Aging in Rhesus Macaques

This is a competing renewal for a program project grant at the University of Wisconsin to investigate the effects of dietary restriction and aging on immune function in rhesus macaques. This small subcontract will pay for 25% of a technician's salary and supplies necessary to produce MHC tetramer reagents.

R01 (subcontract) 10/01/1999-11/30/03 3%
NIH $46,664
AIDS and Opiates: Neuorimmune Correlates of AIDS Pathogenesis

This grant will address two major questions: 1) do opiates influence CTL (or HTL) responses which can, in turn, determine the course of disease after infection; and 2) do certain MHC alleles can influence the course of SIV infection *in vivo*. The PI on the grant is David Watkins at the Wisconsin Regional Primate Research Center.

## Pending

HIV Vaccine Trials Network: SEG 01/01/2000-12/31/2004 5%
NIH $150,000

HIV Vaccine Trials Network: Special Emphasis Group on Cellular Immune Responses
This project is part of a large national network and is designed to develop and validate state of the art assays for measuring cellular immune responses induced in people vaccinated with candidate HIV vaccines.

## PATENTS:

" Compositions and Methods for the Detection, Quantitation, and Purification of Antigen-Specific T Cells." Altman, John; McHeyzer-Williams, Michael; Davis, Mark M. (5,635,363).

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2. Collins, S., **Altman, J. D.**.; Marletta, M. A. (1985). " Development of an affinity chromatography resin for the purification of carcinogen-binding proteins from mouse liver" . *Biochem. Biophys. Res. Commun.*, **129**, 155-162.

3. **Altman, J. D.**; Lipka, J. L.; Kuntz, I. D.; Waskell, L.. (1989). " Identification by Proton Nuclear Magnetic Resonance of the Histidines in Cytochrome *b5* modified by Diethyl Pyrocarbonate" . *Biochemistry*, **28**, 7516-7523.

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5. **Altman, J.D.**; Henner, D; Nilsson, B; Anderson, S; Kuntz, I.D. (1991). " Intracellular expression of BPTI fusion proteins and single column cleavage/affinity purification by chymotrypsin." *Protein Engineering*, , **4**, 593-600.

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11. " Stability of Empty and Peptide-Loaded Class II MHC Molecules at Neutral and Endosomal pH: Comparison to Class I Proteins" , Reich, Z., **Altman, J. D.**, Boniface, J. J., Lyons, D. S., Kozono, H., Ogg, G., Morgan, C., Davis, M. M. *Proc. Natl. Acad. Sci., U.S.A.*, **94**, 2496-2500 (1997).

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13. Murali-Krishna, K., **Altman, J.D.**, Suresh, M., Sourdive, D., Zajac, A., Miller, J. P., Slansky, J., Ahmed, R. " Counting Antigen Specific CD8 T cells: A Re-evaluation of Bystander Activation During Viral Infection" *Immunity* , 8(2), 177-187 (1998) (Murali-Krishna and **Altman** are co-first authors).

14. Gütgemann,I., Fahrer, A. M., **Altman, J. D.**, Davis, M. M., Chien, Y.-h. " Induction of rapid T cell activation and tolerance by systemic presentation of orally administered antigen." , *Immunity*, **8**, 667-673 (1998).

15. Flynn, K. J., Belz, G. T., **Altman, J. D.**, Ahmed, R., Woodland, D. L., Doherty, P. C. " Virus-Specific $CD8^+$ T Cells in Primary and Secondary Influenza Pneumonia." *Immunity*, **8**, 683-691, (1998).

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## Invited Lectures:

" The Kinetics of Antiviral T Cell Immune Responses." Plenary session at the American Association of Immunologists Annual Meeting, May 13, 2000.

" Vaccine induced T cell immune responses in rhesus macaques." University of Toronto, March 7, 2000.

" The Kinetics of Antiviral T Cell Immune Responses." Keystone Meeting on " Cell Biology of Virus Entry, Replication and Pathogenesis", March 3, 2000.

" Preparation and use of MHC Tetramers ." Workshop Meeting of the European Concerted Action 'Correlates of Protective Immunity to HIV Infection And Disease' Group, Würzburg, Germany, February 14-16, 2000.

" Quantitative Analysis of Antiviral T Cell Responses." Rush Presbyterian Medical College, Chicago, IL. January 17, 2000.

" Quantitative Analysis of Antiviral T Cell Responses." Harvard Medical School. December 10, 1999.

" Quantitative Analysis of Antiviral T Cell Responses." Autumn Immunology Conference, Chicago, IL October 21, 1999.

" Application of tetramer technology in the rhesus macaque", Office of AIDS Research/NCRR Workshop on " MHC Typing Issues and Breeding of Genetically Defined Nonhuman Primates for AIDS Vaccine Studies." Bethesda, MD, August 24, 1999.

" Direct ex vivo analysis of antigen-specific T cell responses", NCI/NIAID Workshop on Infectious Etiologies of Chronic Diseases, Rockville, MD, June 14, 1999.

" Quantitative Analysis of Antiviral T Cell Immune Responses." Medical College of Georgia, Augusta, GA, May 20, 1999.

" The New Math of Antiviral T Cell Responses", 1999 Viruses and Cells Gordon Conference, Il Ciocco, Italy, May 6, 1999.

" The MHC Tetramer Core Facility." NIAID Workshop on MHC Tetramers, Washington, DC, April 22, 1999.

" Direct quantitation of cellular immune responses to HIV: New tools to study antiviral immune responses and guide HIV vaccine development." American Federation for Medical Research (part of the 1999 FASEB meeting), Washington DC, April 21, 1999.

" Quantitative Analysis of Antiviral CD8+ T Cell Responses." Wake Forrest University, April 8, 1999.

" What are the T cells stained by tetramers" , Novartis Foundation Discussion Meeting, London, March 12, 1999.

" T Cell Memory" , Symposium Chair, Royal Society Meeting, March 11, 1999.

" Analysis of cross-reactivity in antigen-specific CD8+ T cells by analysis of peptide-stimulated intracellular IFN• production and staining with MHC tetramers," NIH Workshop on Assessing Cytokine Responses in Vaccinated Macaques, Rockville, MD, February 11, 1999.

" Defining T Cell Memory" , Epimmune, January 27, 1999.

" Defining T Cell Memory" , The Scripps Research Institute, January 26, 1999.

" Defining T Cell Memory" , Plenary session presentation at Keystone Conference, Joint Meeting on HIV Vaccine Development and AIDS Pathogenesis, January 8, 1999.

" Massive CD8+ T Cell Responses to Viral Infections" , August 21, 1998, International Symposium on The Major Histocompatibility Complex: From Genes to Function. Queensland, Australia.

" The New Math of Antiviral T Cell Responses," St. Jude Children's Hospital, Memphis Tennessee. July 13, 1998.

" The New Math of Antiviral T Cell Responses," July 27, 1998, Merck Laboratories.

" Direct Identification of Antigen-Specific T Cells" , October 19, 1997, American Society for Histocompatibility and Immunogenetics.

" Evaluation of HIV Specific CD8+ T Cell Responses by Staining with MHC Tetramers" , July 21, 1997, AIDS Clinical Trials Group Meeting.

" Direct Identification of Antigen-Specific T Cells" , May 5, 1997, National Cooperative Vaccine Development Group Conference, National Institutes of Health.

" Direct Identification of Antigen-Specific T Cells" , February 19, 1997, Merck Laboratories.

## Editorial Boards

Virology

## Journal Reviews

Cellular Immunology
Immunity
International Immunology
Journal of Immunology
Journal of Virology
Tissue Antigens
Virology

## Service Activities

Panel Member, Strategic Planning Meeting for NIH Vaccine Research Center, Washington, D.C. April 16-17, 1999.

Panel Member, NIH-sponsored MHC Tetramer Workshop, Bethesda, MD April 22, 1999.

Panel Member, NCI/NIAID-sponsored Workshop on Infectious Etiologies of Chronic Diseases, Rockville, MD., June 13-14, 1999.

Conference Symposium Chair, Autumn Immunology Conference, Chicago, IL. October 1999

## Teaching

Medical Microbiology, Spring 2000. Two lectures on human retroviruses and HIV, given to first year students in the physician assistants program.

Medical Microbiology, Fall 1999. Four lectures on T cell immunity to second year medical students

Current Topics in Immunology. Course supervisor (and discussion leader) for a course given annually in the fall to second year graduate students of the Immunology and Molecular Pathogenesis program at Emory University. 1998-1999.

Advanced Immunology. Annual lecture on measuring T cell responses in a course given in the fall to first year IMP graduate students at Emory University.

Medical Microbiology, Spring 1999. Two lectures on human retroviruses and HIV, given to first year students in the physician assistants program.

Medical Microbiology, Spring 1998. Five lectures on autoimmunity, allergy, hypersensitivities, T cell immune responses, and HIV, given to first year students in the physician assistants program.